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(54) Title: INSECT CONTROL METHOD WITH GENETICALLY ENGINEERED BIOPESTICIDES

(57) Abstract

Insect pests can be controlled by treating the pests or their loci with a combination of recombinant virus and organic insecticide. The recombinant virus preferably is a baculovirus. Combinations of recombinant baculovirus with chemical insecticides provide a dose-response in pests, such as insects, that is greater than additive. Preferred treatments of the invention are uses of recombinant baculoviruses that express a foreign protein or toxin, with pyrethroid insecticides. Treatments against pyrethroid resistant pests are especially useful.

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INSECT CONTROL METHOD WITH
GENETICALLY ENGINEERED BIOPESTICIDES

5   ## Field of the Invention

The present invention generally relates to uses of recombinant expression vectors expressing foreign proteins in controlling insects, and more particularly to a method utilizing insect pathogens,
10   preferably recombinant, in conjunction with synthetic chemical insecticides to magnify insect kill rate.

This invention was made with Government support under Grant No. 5-T32-ES07059, awarded by the National Institutes of Health. The Government has
15   certain rights in this invention.

Background of the Invention

The lepidopteran family Noctuidae includes some of the most destructive agricultural pests, such as the genera *Heliothis*, *Heliocoverpa*, *Spodoptera*, and
20   *Trichoplusia*. For example, included in this family are the tobacco budworm (*Heliothis virescens*), the cotton bollworm (*Heliocoverpa zea*), the cotton leafworm (*Alabama argillacea*), the spotted cutworm (*Amathes c-nigrum*), the glassy cutworm (*Crymodes devastator*), the
25   bronzed cutworm (*Nephelodes emmedonia*), the fall armyworm (*Laphygma frugiperda*), the beet armyworm (*Spodoptera exigua*), and the variegated cutworm (*Peridroma saucia*). Attempts to control these and other insects have often involved the use of pyrethroid

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insecticides.   Wildtype   baculoviruses   have   been   used
with limited commercial success.

Pyrethroid   insecticides   now   dominate   the
insecticide market with sales reaching into the billions
annually.   However, sales are beginning to stalemate due
in part to the wide-scale presence of pest resistance to
these compounds.   In cotton alone, the presence of pyr-R
Heliothis species has begun to result in millions of
lost   dollars   annually.   In   fact,   in   several   cases
pyrethroid   insecticides   have   completely   failed   to
control   infestations   of   *Heliothis*   larvae   in   cotton,
which has resulted in complete destruction of the crop.
Consequently,   there   has   been   an   enormous   effort   to
control   pyrethroid   resistance   in   *Heliothis*   species   in
cotton.

Agricultural   producers   sometimes   attempt   to
restrain their use of pyrethroid insecticides until late
in the growing season as a strategy against pyrethroid
resistance.   As a result, the producers have to turn to
the   less   effective   and   more   expensive   organophosphate
and carbamate insecticides, which have also been plagued
with resistance problems.   Therefore, the development of
a new and effective pesticide to control pyr-R pests
would be extremely valuable in any management strategy.

Resistance of agricultural pests to pesticides
also   leads   to   environmental   and   human   health   risks.
These   problems   arise   because   another   response   by
producers to pyrethroid (and other insecticide) resis-
tance is the use of increasing amounts of pesticide, and
the use of more non-selective and toxic compounds, in
order   to   overcome   pest   resistance.   This   creates   a
destructive and vicious cycle.

Nevertheless, the uses of synthetic chemicals,
such as exemplified by the pyrethroids, are an integral
component   of   modern   agriculture   and   are   probably
necessary to maintain our current level of agriculture

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productivity, although alternative control agents, such as the earlier mentioned recombinant insect pathogens, are being explored for pest control.

Recently, the nuclear polyhedrosis virus *Autographa californica* (AcNPV), from the family Baculoviridae, has been genetically modified for an increased speed of kill by expressing insect-selective toxins. The introduction of insect-selective toxins into an insect-pathogenic virus has resulted in an approximate 30% reduction in the killing time of insect hosts, as is described by U.S. Serial No. 08/229,417, filed April 15, 1994, which is a continuation-in-part application of U.S. Serial No. 07/629,603, filed December 19, 1990, having (in part) common assignment herewith.

Among the insect specific toxins suggested for use in insect control are toxins from *Bacillus thuringiensis* from the scorpions *Buthus eupeus* and *Androctonus australis* and from the mite *Pyemotes tritici*. Further, Hammock et al., (*Nature, 344*, pp. 458-461, 1990) have described use of JHE to control insects, U.S. Serial No. 07/927,851, filed August 10, 1992, and U.S. Patent 5,098,706, issued March 24, 1992, both of common assignment herewith.

A new tool to control resistant, as well as susceptible, insect pest populations would be very desirable.

Summary of the Invention

In one aspect of the present invention, a method for controlling pests is provided. Pests controlled in accordance with the invention are, for example, from the group insects, acarids, and nematodes. Such pests are treated (or their loci treated) with a synergistic combination of recombinant virus and organic insecticide. The recombinant virus preferably is a

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baculovirus that expresses a foreign protein or a functional derivative thereof in pest cells infected with the recombinant baculovirus.

Treatments in accordance with the invention
5   can be simultaneous (such as by applying a pre-mixed composition of recombinant virus and organic insecticide). Alternatively, the pests or loci may first be treated by applying virus followed by organic insecticide within about 24 hours.

10 **Brief Description of the Drawing**

Figure 1 illustrates "before and after" larval exposure pictures of tomato plants to illustrate a property of insect control in conjunction with the use of recombinant baculoviruses.

15 **Detailed Description of the Preferred Embodiments**

The present invention is the use of genetically engineered insect viruses in combination with synthetic, chemical insecticides to treat pests such as insects. Although baculoviruses will be used
20 throughout as an illustration, this invention can be practiced with a variety of insect viruses, including DNA and RNA viruses. Using baculovirus as one example of insect viruses, we have discovered an interaction between the use of recombinant viruses in conjunction
25 with chemical insecticides that can have an effect greater than the single effects. We will first describe suitable recombinant baculoviruses for practicing the invention.

By "baculovirus" insecticides is meant any
30 baculovirus of the family *Baculoviridae*, such as a nuclear polyhedrosis virus (NPV). Baculoviruses are a large group of evolutionarily related viruses, which infect only arthropods; indeed, some baculoviruses only infect insects that are pests of commercially important

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agricultural and forestry crops, while others are known that specifically infect other insect pests. Because baculoviruses infect only arthropods, they pose little or no risk to humans or the environment.

5   Of the suitable DNA viruses, in addition to the *Baculoviridae* are the entomopox viruses (EPV), such as *Melolontha melonotha* EPV, *Amsacta moorei* EPV, *Locusta migratoria* EPV, *Melanoplus sanguinipes* EPV, *Schistocerca gregaria* EPV, *Aedes aogypti* EPV, and 10 *Chironomus luridus* EPV. Other suitable DNA viruses are granulosis viruses (GV). Suitable RNA viruses include togaviruses, flaviviruses, picornaviruses, cytoplasmic polyhedrosis viruses (CPV), and the like. The subfamily of double stranded DNA viruses *Eubaculovirinae* includes 15 two genera, NPVs and GVs, which are particularly useful for biological control because they produce occlusion bodies in their life cycle. Examples of GVs include *Cydia pomonella* GV (coddling moth GV), *Pieris brassicae* GV, *Trichoplusia ni* GV, *Artogeia rapae* GV, and *Plodia* 20 *interpunctella* GV (Indian meal moth).

          Suitable baculoviruses for practicing this invention may be occluded or non-occluded. The nuclear polyhedrosis viruses ("NPV") are one baculovirus sub-group, which are "occluded." That is, a characteristic 25 feature of the NPV group is that many virions are embedded in a crystalline protein matrix referred to as an "occlusion body." Examples of NPVs include *Lymantria dispar* NPV (gypsy moth NPV), *Autographa californica* MNPV, *Anagrapha falcifera* NPV (celery looper NPV), 30 *Spodoptera litturalis* NPV, *Spodoptera frugiperda* NPV, *Heliothis armigera* NPV, *Mamestra brassicae* NPV, *Choristoneura fumiferana* NPV, *Trichoplusia ni* NPV, *Heliocoverpa zea* NPV, and *Rachiplusia ou* NPV. For field use occluded viruses are preferable due to their greater 35 stability since the viral polyhedrin coat provides protection for the enclosed infectious nucleocapsids.

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Among illustrative, useful baculoviruses in
practicing this invention are those *Anagrapha falcifera*,
Anticarsia gemmatalis, *Buzura suppressuria*, *Cydia
pomonella*, *Heliocoverpa zea*, *Heliothis armigera*,
5   *Manestia brassicae*, *Plutella xylostella*, *Spodoptera
exigua*, *Spodoptera littoralis*, and *Spodoptera litura*.
A particularly useful "NPV" baculovirus for practicing
this invention is AcNPV, which is a nuclear polyhedrosis
virus from *Autographa californica*. *Autographa cali-
10   fornica* is of particular interest because various major
pest species within the genera *Spodoptera*, *Trichoplusia*,
and *Heliothis* are susceptible to this virus.

Such baculoviruses may be genetically
engineered into more potent insecticidal forms by
15   cloning genes encoding a foreign protein such as insect
toxin into the genome of the baculovirus. Illustrative
toxins are from the scorpions *Buthus eupeus* and
Androctonus australis and from the mite *Pyemotes
tritici*.

20   The expressed foreign protein (which may be a
glycoprotein) preferably is an insecticidal toxin,
particularly an arthropod or other invertebrate toxin,
such as a scorpion toxin, a wasp toxin, a snail toxin,
or a spider toxin. A useful scorpion toxin is, for
25   example, that from *Androctonus australis*. A useful
snail venom is that from the snail conotoxins (cone
shell snail poisons), which the animal delivers by mouth
and some individual toxins of which appear to be
selective for arthropods including insects. See, for
30   example, Olivera et al., "Diversity of *Conus* Neuropep-
tides," *Science*, *249*:257-263 (1990).

The amino acid sequence of the excitatory
toxin from *Androctonus australis* (AaIT), was determined
and the sequence published in Darbon 1982. The AaIT
35   toxin exhibits toxicity to insects, while being non-
toxic to isopods and mammals.

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Various other scorpion toxins such as of the Buthoid scorpion can also be used, such as LqqIT2, which is a depressive insect toxin from *Leiurus quinquestriatus quinquestriatus*. The purification method used to obtain this neurotoxin was published by Zlotkin et al., *Archives of Biochem. Biophys.*, *240*:877-887 (1985).

BjIT2 is another depressive insect toxin and is from *Buthotus judaicus*. The purification has been published in Lester et al., *Biochim. Biophys. Acta*, *701*:370-381 (1982). BjIT2 exists in two isoforms which differ in amino acid sequence at position 15. Form 1 has isoleucine in this position while form 2 has valine.

LqhIT2 is yet another depressive insect toxin from *Leiurus quinquestriatus hebraeus* which was purified using reverse phase HPLC.

An "intermediate" toxin has also been discovered which affects insect sodium channels in a manner very similar to the effect of alpha toxins on mammalian sodium channels. This neurotoxin was derived from a yellow scorpion *Leuirus quinquestriatus hebraeus*, Buthinae, Buthidae and is called herein LqhP35. The identification and purification of this toxin was described in "Toxin to Insects Derived from the Venom of the Scorpion *Leiurus quinquestriatus hebraeus*," published by Citan et al., *Biochemistry*, *29*:5941-5947 (1990), renamed "LqhαIT."

Other toxins, purified from the venom of the chactoid scorpion, *Scorpio maurus palmatus*, can also be used. For example, SmpIT2, from the chactoid scorpion, *Scorpio maurus palmatus*, is a depressive insect toxin. Its purification is described in Lazarovici et al., *J. Biol. Chem.*, *257*:8397-8404 (1982).

Still other toxins purified from the venom of the chactoid scorpion, *Scorpio maurus palmatus*, are SmpCT2 and SmpCT3, and crustacean toxins, whose purification has been described in Lazarovici, Ph.D. thesis

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(1980), Hebrew University, Jerusalem, "Studies on the Composition and Action of the Venom of the Scorpion *Scorpio maurus palmatus* (Scorpionidae)."

5   For producing recombinant baculoviruses for the purpose of controlling insects, a secretion signal sequence is preferably included.   Secretion signal sequences may be derived from proteins of bacteria, yeast, fungi, or higher eukaryotes, including both animals and plants (for examples, see Watson, *Nucl. Ac.*

10   *Res.*, *12*:5145-5164 (1984).   More preferred are secretion signal sequences from proteins of insect origin, for example those of cecropin B from *Hyalophora cecropia* (van Hofsten et al., *PNAS*, *82*:2240-2243 (1985)), and the eclosion hormone from *Manduca sexta* (Horodyski et al.,

15   *PNAS*, *86*:8123-8127 (1989)).   Also preferred are the secretion signal sequences naturally associated with scorpion toxins, which can be determined by the analysis of mRNA, cDNA, or genomic DNA.   More preferred is the natural secretion signal sequence of AaIT (Bougis et

20   al., *J. Biol. Chem.*, *264*:19259-19265 (1989)).

        Foreign proteins, or toxins, may be expressed as functional derivatives of the toxin.   A "functional derivative" of the toxin is a compound which possesses a biological activity (either functional or structural)

25   that is substantially similar to a biological activity of the toxin.   The term "functional derivative" is intended to include the "fragments," "variants," "analogues," or "chemical derivatives" of a molecule. A "fragment" of a molecule such as the toxin is meant to

30   refer to any polypeptide subset of the molecule.   A "variant" of a molecule such as the toxin is meant to refer to a molecule substantially similar in structure and function to either the entire molecule, or to a fragment thereof.   A molecule is said to be "substan-

35   tially similar" to another molecule if both molecules have substantially similar structures or if both

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molecules possess a similar biological activity. Thus,
provided that two molecules possess a similar activity,
they are considered variants as that term is used herein
even if the structure of one of the molecules is not
5   found in the other, or if the sequence of amino acid
residues is not identical. An "analog" of a molecule
such as the toxin is meant to refer to a molecule
substantially similar in function to either the entire
molecule or to a fragment thereof. As used herein, a
10 molecule is said to be a "chemical derivative" of
another molecule when it contains additional chemical
moieties not normally a part of the molecule.

Such moieties may improve the molecule's
solubility, absorption, biological half-life, etc.
15 Moieties capable of mediating such effects are disclosed
in Remington's Pharmaceutical Sciences (1980). Proce-
dures for coupling such moieties to a molecule are well
known in the art.

Expression of the toxin will, in general,
20 include a promoter region sufficient to direct the
initiation of RNA synthesis. One baculovirus gene is
that coding for polyhedrin, since the polyhedrin protein
is one of the most highly expressed eucaryotic genes
known. Thus, the polyhedrin promoter is preferred,
25 although other promoter and hybrid promoter sequences
may be used.

As will be further exemplified, a particular
recombinant baculovirus has been constructed from
Autographa californica by cloning a gene encoding the
30 insect toxin from *Androctonous australis* (Hector) into
the AcNPV genome, with the toxin being expressed under
polyhedrin promoter control. (Such a construction is
also described by U.S. Serial No. 08/229,417, already
referenced and *Bio/Technology*, *9*:848-852, 1991.) We
35 designate this particular, illustrative recombinant
baculovirus embodiment as "AcAaIT," but note that some

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persons in the art have utilized the designation "HIT,"
instead of "IT," for the toxin.   (See, for example,
Loret et al., *Biochemistry*, *29*, pp. 1492-1501 (1990),
who described several of the active neurotoxins from the
5   scorpion as "*Androctonous australis* Hector.") We have
used this recombinant baculovirus (such as in comparison
with the wild-type AcNPV designated as "wtAcNPV"), as
will be hereinafter more fully described. However, it
should be understood that the invention is not limited
10   to the particular recombinant baculovirus AcAaIT used to
illustrate aspects of this invention, although AcAaIT is
particularly preferred.

        Therefore,   the   just   described   recombinant
baculoviruses are one part, or aspect, of practicing
15   this invention, while the pests treatable in accordance
with the present invention are from the group insects,
acarids, and nematodes.   For example, illustrative
insect pests that can be treated are the tobacco
budworm, the cotton bollworm, the cotton leafworm, the
20   spotted cutworm,   the glassy cutworm,   the bronzed
cutworm, the fall armyworm, the beet armyworm, and the
variegated cutworm.   The other part, or aspect, of
practicing this invention is using such already
described recombinant baculoviruses in conjunction with
25   chemical insecticides.

        The synthetic, organic insecticides with which
the present method may be practiced include: Na^+
channel agonists (i.e. pyrethroids), Na^+ channel
blocking agents (i.e. pyrazolines), acetylcholinesterase
30   inhibitors (i.e. organophosphates and carbamates),
nicotinic   acetylcholine   binding   agents   (e.g.
imidacloprid), gabaergic binding agents (e.g. emamectin
and fipronil), octapamine agonists or antagonists (i.e.
formamidines), and oxphos uncouplers (e.g. pyrrole
35   insecticides).

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As will be hereinafter further exemplified in the "Experimental" section, we have used low rates of cypermethrin (a Type II pyrethroid) or allethrin (a Type I pyrethroid) in combination with wild-type AcNPV (wtAcNPV) or AcAaIT (>LC_{99}) in larvae of *H. virescens* to illustrate the invention.

The combination of the recombinant AcAaIT (even with low rates) of either pyrethroid noted above produced a dose-response greater than potentiation. Thus, use of AcAaIT in accordance with the invention in combination with allethrin or cypermethrin resulted in 54.8 and 64.6% reductions in the LT_{50}s, respectively, of the insect larvae when compared to wtAcNPV. These data illustrate a surprising property and an advantage for the invention. That is, pyrethroids and AaIT interact in a manner which magnifies the rate of kill beyond potentiation, and are synergizing (effect greater than the algebraic sum of the single effects) the rate of kill by AcAaIT. An even more surprising result is that the resistant strain of *H. virescens* is more sensitive to the recombinant baculovirus when compared with an insecticide-susceptible strain, demonstrating the potential utility of this invention.

We believe, without being bound by theory, that a simultaneous exposure of AaIT and pyrethroid results in a synergistic interaction at the sodium channel. It appears that the toxins that display synergistic activity in combination with recombinant baculovirus can be predicted based on the mechanism of action. As expected based on their activity as acetylcholinesterase inhibitors, organophosphates and carbamates were also found to be synergistic.

Thus, in practicing the invention, pests being controlled are treated (and/or their loci treated) with a combination of recombinant baculovirus and an organic insecticide predicted to be synergistic based on its

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mechanism of action. The combination may conveniently be applied for treating the pests as a single admixture.

As is well known, organic insecticides may be applied by means such as spraying, atomizing, dusting, scattering or pouring and may be formulated for such applications as powders, dusts, granulates, as well as encapsulations such as in polymer substances. When practicing this invention such conventional application means may be used. Preferably, the organic insecticide and recombinant baculovirus will be admixed in desired proportions, and may typically include inert carriers such as clay, lactose, defatted soy bean powder, and the like to assist in application.

However, it is possible to apply compositions including each component separately, by utilizing the baculovirus first then followed (preferably within about forty-eight hours) by the organic insecticide. When the baculovirus is first used (followed by organic insecticide), then the baculovirus can be applied by conventional means, such as spraying. An advantage of applying the baculovirus first is due to a "falling off" property described hereinafter in Example 3.

EXPERIMENTAL

EXAMPLE 1

The recombinant AcAaIT that was used in performing experiments was constructed as recombinant transfer vector pAcUW2(B)·AaIT. This is orally infectious and expresses an insect-selective toxin (AaIT), which is isolated from the scorpion *Androctonus australis*, and is under the control of the p10 promoter. This construction was earlier reported in *Bio/Technology*, *9*:848-852 (Sept. 1991). Briefly, the AaIT toxin gene (sometimes also herein designated

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"AaHIT") in the transfer vector, pBK283, was digested
with SacI and XbaI to excise the complete AaIT gene
including a signal sequence of bombyxin required for
secretion. The DNA fragment was inserted into the
5   pTZ-18R plasmid between the SacI and XbaI sites. The
plasmid was cleaved with SacI where a synthesized
SacI-BamHI-SacI linker was inserted. The resulting
plasmid contained two BamHI sites; one site near the 5'
end of the toxin cDNA and the other site between the
10   stop codon, and XbaI site of the original fragment. The
insertion of the linker and AaIT gene was confirmed by
1% agarose gel electrophoresis by digestion with BamHI
and screening for the resultant 300 base pair fragment.
The protocol for GeneClean (BiolO1) was followed in
15   order to isolate the toxin fragment for insertion in the
transfer vector with approximately 30% recovery of the
DNA fragment.

The excised AaIT gene fragment was ligated
into the BglII cloning site of the digested and
20   dephosphorylated plasmid vector pAcUW2(B), and then
transformed in the JM 101 strain of *E. coli*. As a
result of the ligation, both the BglII and BamHI sites
were eliminated, and resultant plasmids were screened
for a unique SacI site. Three SacI positive clones were
25   identified from sixty colonies using 1% agarose gel
electrophoresis. The direction of insertion was
confirmed by double digestion with SacI (5' end of the
AaIT gene) and BamHI (within the coding sequence of the
polyhedrin gene of the transfer vector). Two of three
30   recombinant transfer vectors were carrying the AaIT gene
in the correct orientation and had the approximate 1.6
kbp fragment inductive of the correct orientation. The
construction resulted in the AaIT cDNA sequence now
inserted downstream of a duplicated p10 protein promoter
35   and upstream of the polyhedrin gene 5 producing the
recombinant plasmid pAcUW2(B)·AaIT.

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Isolation of Recombinant AcNPV.

Spodoptera frugiperda cells (Sf-9) were propagated in ExCell 400 media (JR Scientific) supplemented with 2.5% fetal bovine serum. Sf-9 cells were
5   cotransfected by calcium precipitation with the plasmid pAcUW2(B) and polyhedrin negative AcNPV DNA. Polyhedrin-infected cells were identified and collected at 5 days post infection. The recombinant virus was plaque purified by screening for the polyhedrin positive
10 plaques. Purification of the recombinant virus was expedited by sodium dodecyl sulfate (SDS) treatment (1%) of the cells after each plaque purification in order to eliminate polyhedrin-negative, non-recombinant virus. After purification of the recombinant virus by the
15 initial plaque assay, individual plaques were purified in 3 successive rounds, and the resultant, pure recombinants were propagated and stored at 4°C and -80°C.

Screening the Recombinant AcUW2(B)·AaIT for Biological Activity.

20   After purification of the recombinant virus by the initial plaque assay, a total of six plaques were isolated and suspended in 500 μl of ExCell media with 2.5% fetal bovine serum. Sf-9 cells in 35 mm dishes (75% monolayer) were inoculated with 100 μL ($\sim$10^6 plaque
25 forming units) of the suspension, and cells were collected 7 days post infection. The individual collections were centrifuged at 1000 g for 5 minutes, and the resulting supernatant was collected and separated from the pelleted cells. The cells were
30 resuspended in double-distilled H_2O, treated with 1% SDS, then vortexed for 5 minutes and washed 3 times. Crude estimates of the plaque forming units per ml were determined from the supernatant.

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EXAMPLE 2

Detailed mortality studies were conducted with 2nd instar *H. virescens* larvae, as more fully described in the Experimental Protocol of the earlier cited paper
5   (*Bio/Technology*, Vol. 9).   Lethal times (LTs) were derived when 2nd instar *H. virescens* larvae were treated with the recombinant and wild-type AcNPV using a common dose of 250 polyhedrin inclusion bodies (PIBs) per plug of diet.

10   Table 1 shows the dose response and mortality times of second instar *H. virescens* larvae for treatments with AcUW2(B)·AaIT (AcAIT) and wild-type AcNPV.

TABLE 1

	LD (PIBs)			LT (hours)		
Treatment	10	50	90	10	50	90
AcUW2(B)·AaIT	1.56	13.3	113	59.8[a]	88.0[a]	129[a]
Wild-Type AcNPV	2.72	21.9	175	91.4	125	172

[a] Significantly different from other treatments – POLO probit analysis program (C.I. 0.95)
20   PIBs = polyhedrin inclusion bodies
LT = lethal time; LD = lethal dose

These data demonstrate the ability of the AcUW2(B)·AaIT to kill *H. virescens* larvae significantly
25   more quickly than the wild-type AcNPV.

EXAMPLE 3

Larvae infected with the recombinant AcAaIT typically started showing symptoms of paralysis and stopped feeding 10 to 15 hours prior to death.   As a
30   result, these larvae can functionally be considered dead, and treatment with AcAaIT represents an approxi-

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mately 40% reduction in the time required to kill host
larvae when compared to wild-type AcNPV.

We designed experiments to assess the
differences in feeding damage that would be incurred by
5   tomato plants infested with larvae of *H. virescens* that
had been infected with uninfected controls wild-type
AcNPV or AcAaIT. Individual plants were infested with
two 4th instar larvae that were uninfected (control, I)
or had previously been infected with wild-type AcNPV
10   (II) or /AcAaIT (III) 48 hours earlier. These larvae
were allowed to feed continuously on the plants until
death.

The AcAaIT treatment resulted in significantly
less damaged plants. However, what is not evident is
15   the fact that the larvae infected with AcAaIT were
unable to inflict more damage on the tomato plants
because they were falling from the plant. In fact, we
observed five of eight 4th and 5th instar larvae
infected with AcAaIT had fallen from the plant at 120
20   hours post infection. These individuals were apparently
unable to crawl back up the plant due to the onset of
paralysis and is sometimes referred to as the "falling
off" property. This is illustrated by Fig. 1.

In Fig. 1, panels A illustrate the tomato
25   plants before exposure to any larvae. Panel IA thus
shows the control "before" larvae while panel IB shows
the control after larvae without any virus had been
freely feeding on the plant until the experiment was
stopped.   As   demonstrated   by   the   illustrations
30   (reproduced from photographs), the control tomato plants
were badly ravaged by the larvae. Turning to panel IIA,
we again see tomato plants before introduction of
larvae. In panel IIB larvae infected with wild-type
AcNPV have been feeding on the plants for about 190-200
35   hours at which point the larvae had died and the
experiment (for all panels IB, IIB, and IIIB) was

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stopped.   While   panel   IIB   showed   considerable
improvement over the panel IB control, the plant
nevertheless had been subjected to up to about 200 hours
of feeding activity before the larvae died.   However,
5   turning to panels IIIA (the beginning of the experiment)
and IIIB (the end), we see that the panel IIIB plants
have considerably more foliage when the experiment was
stopped.   These plants have more foliage because the
inventive baculovirus AcAaIT infected larvae had their
10   behavior disrupted before death so that five of eight
larvae fell off the plant after 120 hours of feeding and
the remaining three fell shortly thereafter.

         Table 2 confirms that the insects become
irritated and were not feeding on the food substrate or
15   were wandering off the substrate a significant amount of
time.

TABLE 2

Percentage of Time During Course of Infection
Tobacco Budworm Larvae Were Off the Food Substrate

Food Substrate	AcAaIT	Wild Type Virus
Cotton	80.40%	13.30%
Romaine	64.20%	15.40%
Iceberg	85.90%	13.20%
Diet	59.90%	11.00%

         To obtain the data summarized by Table 2, the
larvae were droplet fed virus at 2,000 PIBs/μl as
neonates and checked every 6 to 8 hours.   Larvae were
scored as to whether they were off or on the food
30   substrate as summarized in Table 2 above.   At 64 hours
post-infection, over 57% of all tobacco budworm larvae
were not on the substrate, versus 32% of wild-type
infected insects (which were not yet expressing
symptoms).   The purpose of this experiment was to
35   confirm that larvae infected with AcAaIT before they die

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become paralyzed (or have their feeding behavior disrupted), while this does not happen with wild-type infected insects.

It is plausible that low levels of the
5   neurotoxin, AaIT, early in the infection process may irritate the larval host, thus resulting in the "falling off" or eating disruption phenomena. Even though the larva is still potentially capable of feeding and causing damage, it no longer has access to the plant.
10 With the reduced killing time and early immobilization of the host larva by paralysis, AcAaIT appears effectively to curtail food consumption which may translate into reduced feeding damage.

EXAMPLE 4

15   The slow time to death and resulting crop damage is often cited as a major limitation to the commercial success of viral insecticides. Twenty-four to forty-eight hour mortality is a common target for classical insecticides. Thus, in this study we have
20 concentrated on studying speed of kill expressed as lethal times ("LTs"). Analogous approaches can be used to determine lethal doses, which are likely to be of major economic importance.

As is summarized by Tables 3A and 3B, we
25 investigated the interaction of low rates (LC_{10}-LC_{20} at 24 h; Table 4) of six insecticides in combination with wild-type AcNPV or AcAaIT (>LC_{99}) in neonate larvae of *H. virescens*. For example, two of the compounds studied were pyrethroids, allethrin (Type I pyrethroid). In our
30 study we found a subadditive response when low rates of allethrin or cypermethrin were combined with wild-type AcNPV. Thus, a combination of allethrin and wild-type AcNPV reduced the LT_{50} to 71.9 h (53.1 h, theoretical additive effect), which is a 17.9% reduction when

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compared to wild-type AcNPV alone. Likewise, cypermethrin in combination with wild-type AcNPV produced a LT_{50} of 65.8 h (52.1 h, theoretical), which represents a 24.2% reduction when compared to wild-type AcNPV. These results suggest that the pyrethroids and the wild-type AcNPV act independently of one another, but result in a subadditive response since the combination is positive although it is significantly less than a theoretical interaction (additive effect).

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TABLE 3A

Insecticides Combined with Wild-Type and Recombinant Baculoviruses

Compound	Common Name	Type	Mode of Action	Source
Allethrin	Cinerine, Alleviate, Bioallethrin	Pyrethroid Type I	Sodium Channel Agonist	Roussel Uclaf Div. Agrovetennaire, Paris, France
Cypermethrin	Ammo, Cymbush, Cynoff, Cyperkill, Fenom, Arrivo	Pyrethroid Type II	Sodium Channel Agonist	ICI Americas (Zeneca) Goldsboro, NC
DDT	DDT	Chlorinated Hydrocarbon	Sodium Channel Agonist	Synthesis by B.D. Hammock, UC Davis, CA
Endosulfan	Thiodan, Cyclodan, Thiosulfan, Malix, Thionex, Tiovel	Cyclodiene	GABA Channel Agonist	FMC Corp. Ag Chemical Group Middleport, NY
Methomyl	Lannate, Nudrin	Carbamate	AChE Inhibitor	E.I. DuPont de Nemours & Co. Wilmington, DE
Profenofos	Curacon, Polycron, Selecron	Organo-phosphate	AChE Inhibitor	Ciba-Geigy Greensboro, NC

GABA, g-aminobutyric acid; AChE, acetylcholinesterase

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TABLE 3B

Time-Response of Neonate Larvae of Tobacco Budworm to Recombinant or Wild-Type AcNPV with Low Concentrations of Classical Insecticides

	Treatment[a]	n	Slope ± SE	LT_{10} (h; 95% CL)	LT_{50} (h; 95% CL)	LT_{90} (h; 95% CL)
5	Wt AcNPV	204	9.26 ± 0.41	64.9 (59.0 - 69.7)	89.8 (85.0 - 93.4)	123 (115 - 134)
	AcAaIT	276	7.73 ± 0.25	47.9 (44.8 - 50.6)	70.2 (68.0 - 72.4)	103 (98.3 - 109)
	AcJHE.KK	90	7.14 ± 0.53	50.5 (24.1 - 62.7)	76.3 (60.8 - 99.7)	115 (91.5 - 266)
	Allethrin	114	1.46 ± 0.19	17.3 (11.5 - 22.5)	132 (106 - 185)	1000 (552 - 2680)
10	Allethrin + AcAaIT	125	3.54 ± 0.20	17.5 (12.6 - 21.7)	40.2 (34.9 - 45.5)	92.5 (78.2 - 118)
	Allethrin + WtAcNPV	119	2.31 ± 0.19	20.0 (16.1 - 23.5)	71.9 (65.5 - 80.0)	258 (205 - 354)
	Cypermethrin	110	1.49 ± 0.24	17.1 (7.99 - 24.8)	124 (97.5- 195)	902 (436 - 4190)
15	Cypermethrin + AcAaIT	112	3.19 ± 0.27	12.5 (5.60 - 18.5)	31.5 (22.7 - 38.1)	79.0 (65.3 - 111)
	Cypermethrin + WtAcNPV	109	2.09 ± 0.23	16.0 (10.9 - 20 7)	65.8 (59.3 - 73.8)	270 (203 - 418)
	Cypermethrin + AcJHE.KK	83	3.81 ± 0.27	24.2 (18.7 - 28 8)	52.5 (47.3 - 58.0)	114 (97.8 - 142)
20	DDT	80	1.45 ± 0.30	12.6 (3.90 - 23 8)	96.7 (73.5 - 165)	740 (318 - 10320)
	DDT + AcAaIT	80	3.12 ± 0.32	16.0 (7.84 - 23 0)	41.2 (31.4 - 48.7)	107 (84.6 - 160)
	DDT + WtAcNPV	80	2.25 ± 0.29	17.7 (5.78 - 28 2)	66.0 (49.9 - 84.6)	245 (159 - 688)
	Endosulfan	80	1.51 ± 0.27	10.4 (3.75 - 17 3)	73.3 (61.0 - 90.3)	514 (296 - 1580)
25	Endosulfan + AcAaIT	80	4.23 ± 0.35	23.8 (16.5 - 29 7)	47.7 (40.6 - 54.2)	95.4 (81.6 - 121)
	Endosulfan + WtAcNPV	80	2.57 ± 0.30	24.7 (14.5 - 33.0)	78.1 (66.7 - 93.6)	247 (176 - 460)
	Methomyl	125	0.69 ± 0.20	4.84 (0.12 - 13 1)	358 (182 - 4070)	26563 (2870-1.25×10^8)
30	Methomyl + AcAaIT	125	3.27 ± 0.28	13.7 (9.16 - 17.8)	34.3 (29.2 - 38.8)	85.7 (73.3 - 108)
	Methomyl + WtAcNPV	125	2.37 ± 0.22	14.6 (8.34 - 20.2)	50.3 (42.5 - 58.1)	173 (132 - 276)
	Profenofos	150	0.76 ± 0.19	8.10 (2.61 - 17.5)	401 (260 - 676)	19810 (9472-1.88×10^4)
35	Profenofos + AcAaIT	150	3.81 ± 0.23	24.5 (20.3 - 28.2)	53.2 (49.2 - 57.1)	115 (103 - 133)
	Profenofos + WtAcNPV	150	2.33± 0.19	16.5 (10.8 - 21.6)	58.5 (52.0 - 65.2)	208 (163 - 302)

40   [a] Dose-response curves were developed for the classical insecticides using neonate larvae of *H. virescens*. Neonate larvae infected with NPVs were exposed to 2000 polyhedrin inclusion bodies (PIBs; $\geq LC_{99}$) using a droplet feeding assay. Three NPVs were utilized including wtAcNPV, AcAaIT, and AcJHE.KK. Larvae which were to be combined with an insecticide were immediately transferred to vials treated with a LC_{10}-LC_{20} (24 h) of the insecticide. Mortality was recorded at 8 to 12 h intervals, and the data were analyzed using a log-probit analyses program (POLO-PC).

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However, as shown by the data of Table 5, the combination of the recombinant AcAaIT with low rates of either pyrethroid produced a dose-response greater than an additive response. Thus, we observed that AcAaIT in

5 combination with allethrin or cypermethrin resulted in 30.0 and 38.7% reductions in the LT_{50}s, respectively, when compared to AcAaIT alone. The LT_{50}s for the combination of AcAaIT and allethrin or cypermethrin were 40.2 h and 31.5 h post infection, respectively (Table

10 3). Both of these LT_{50}s are lower than the theoretical (additive effect) LT_{50}s of 45.8 h and 44.8 h post infection, respectively. These data suggest that the pyrethroids and AaIT interacted in a manner, which magnifies the rate of kill beyond potentiation and are

15 synergizing (supplemental synergism - quicker effect than the algebraic sum of the single effects) the rate of kill by AcAaIT.

Another recombinant virus, AcJHE.KK, was used as a control for the recombinant NPV, AcAaIT. AcJHE.KK

20 expresses a modified version of juvenile hormone esterase, an insect-derived enzyme important in the regulatory development of many lepidopterous insects. The modified JHE has been shown to be insecticidal to several lepidopterous insects (Bonning and Hammock,

25 1994). Although the mode of action of JHE.KK is not yet fully understood, we can assume it does not interfere with the nervous system of insects. We found a 23.8% reduction in the LT_{50} when AcJHE.KK was combined with cypermethrin. The LT_{50} for this combination was 52.5 h

30 which is significantly higher than 47.2 h predicted for the theoretical combination and indicates a subadditive not a supplemental effect (Table 5). Since AcJHE.KK has a LT_{50} similar to that of AcAaIT these data indicate that the pyrethroids do not synergize indiscriminately

35 any baculovirus with enhanced speed of kill. Thus, these data provide further support for a supplemental

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synergistic relationship between the recombinant virus, AcAaIT, and pyrethroid insecticides.

As further shown by Table 3B, another sodium channel agonist, DDT, was also screened for interactions

5   with the NPVs. A combination of DDT and wild-type AcNPV resulted in a 24% reduction in the LT_{50} (66.0 h) when compared to wild-type AcNPV alone (Table 3B). Our data indicate that this combination produced a subadditive effect (46.7 h, theoretical). DDT combined with AcAaIT

10 resulted in a 29.0% reduction in the LT_{50} (41.2 h) when compared to AcAaIT alone. The results of this combination suggest a purely additive response (theoretical $\approx$ actual) since the theoretical time has a very similar LT_{50} of 40.8 hours.

15   Yet another insecticide, endosulfan (a cyclo-diene), was also combined with the NPVs to explore any interactions. Interestingly, our data indicate an antagonistic interaction when endosulfan was combined with the wild-type AcNPV. The LT_{50} for the combination

20 was 78.1 h which was later than the 73.3 h produced by the insecticide alone indicating an antagonistic response. However, the combination of AcAaIT with endosulfan resulted in an LT_{50} of 47.7 h, which is significantly quicker than either AcAaIT or endosulfan

25 alone and indicates a subadditive response.

Yet additional studies were performed with a carbamate (methomyl) or an organophosphate (profenofos) insecticide in combination with AcNPV or AcAaIT. Interestingly, both insecticide and wild-type AcNPV

30 combinations resulted in supplemental synergism. The derived LT_{50} for methomyl and wild-type AcNPV was 50.3 h compared to 71.9 h for the theoretical response (Table 3). Likewise, profenofos in combination with wild-type AcNPV resulted in a LT_{50} of 58.5 h versus 73.5 h for the

35 theoretical additive response. Similarly, the combinations of methomyl or profenofos with AcAaIT resulted in

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supplemental synergistic responses.   The LT_{50}s for
methomyl or profenofos with AcAaIT were 34.3 h and 53.2
h, respectively. The combination of AcAaIT and methomyl
show a 61.8% quicker rate of kill than wild-type AcNPV
5   alone, and a 36.0% quicker rate than AcAaIT alone.
Profenofos and AcAaIT result in a 40.8 and 17.0%
reduction in time of kill compared to wild-type AcNPV
and AcAaIT, respectively.

_____

10   Overall, our data indicate that the pyrethroid
insecticides and ACHE inhibitors appear to provide the
greatest chance of synergizing the recombinant virus,
AcAaIT, in the field and for this reason, use of the
recombinant AcAaIT is particularly preferred. However,
15   other recombinant strains of baculovirus expressing the
AaIT gene would be also preferred (i.e. HzAaIT or
SfAaIT). For the pyrethroids these data suggest that a
simultaneous exposure of AaIT and pyrethroid may result
in a synergistic interaction at the insect sodium
20   channel.   Based on these results, varying rates of
pyrethroids in combination with AcAaIT or other
recombinant viruses expressing AaIT should be tested for
efficacy in the field. The low rates of pyrethroids we
are suggesting should have minimal effect on beneficial
25   arthropods, thus not compromising the host specificity
provided by the virus. The combination is believed also
useful in resistance management strategies.
          Both carbamate and organophosphate insecti-
cides act as poisons at the peripheral nervous system.
30   These compounds inhibit the enzyme, acetylcholinester-
ase, resulting in an accumulation of acetylcholine in
the synapse regions of nerves. This accumulation of
acetylcholine produces a plethora of neurological
responses resulting in a paralysis of the insect.
35   Therefore, it could be suggested that the simultaneous

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action of the insecticide and the peptide toxin would
result in a supplemental synergistic response. It is
difficult to account for the contribution, if any, made
by the virus itself. However, NPVs can infect nerve
5   cells, and eventually the infection results in lysis of
the cell. One could envision that the lysis of nerve
cells by NPV infection would result in an increase of
neurotransmitters in the peripheral nervous system which
could lead to neuromuscular abnormalities. This action
10   might account for the supplemental synergism observed
with both wild-type and recombinant NPVs when combined
with methomyl or profenofos.

As indicated previously, these studies have
emphasized the effects of combinations of viral and
15   chemical insecticides on LT_{50}, but the same approaches
could be applied to dose as well as time. We used here
approximate LD_{10}s (24 h) of the pesticides reported here
to minimize the symptomology and mortality resulting
directly from the chemical pesticide (Table 4).   In
20   practice a variety of factors would influence the rates
used, including the cost and availability of the
biological   and   chemical   pesticide,   environmental
concerns, level of resistant populations in the field,
and other pest management considerations.

25   **EXAMPLE 5**

In this study we compared the LTs of wild-type
and AcAaIT against pyrethroid-susceptible and -resistant
(PEG) larvae of *H. virescens*. The response (LTs) of the
susceptible and PEG neonate larvae to wild-type AcNPV
30   and AcAaIT were very comparable. Statistical analysis
(POLO-PC) of the data indicated no significant
difference in the slopes (PEG = 9.51, susceptible =
9.82, 95% CL) or intercepts.   However, when these
viruses   were   tested   for   effectiveness   against

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pyrethroid-resistant larvae our results lead to the
surprising results as follows. Analysis of the data
indicated a significant difference between the slopes
(PEG = 13.9, susceptible = 7.73, 95% CL) and intercepts
5   for wild-type and AcAaIT, but our results indicated that
the resistant strain, not the susceptible strain, was
killed in a more timely manner. The LT_{90} for the
Stoneville strain was 103 h post infection versus 78.1
h for the PEG strain representing a highly significant
10   24.1% quicker rate of kill against the resistant strain.
        These results indicate that a recombinant
baculovirus such as AcAaIT may be more effective against
pyrethroid-resistant larvae than pyrethroid-susceptible
larvae of *H. virescens* in field situations and could be
15   used as an effective agent to combat pyrethroid
resistance.

        Thus, one aspect of the invention is use of
AcAaIT to control outbreaks of pest insects resistant to
20   pyrethroids. Furthermore, AcAaIT could be integrated
into a resistance management strategy to delay or
prevent pyrethroid resistance in the field. In addition
to reducing resistant populations, this strategy should
extend the efficacy of the pyrethroid insecticides.

25   **EXAMPLE 6**

        We investigated the interaction of low rates
(LC_{10}-LC_{20} at 24 h, Table 4) of several insecticides in
combination with wild-type AcNPV or AcAaIT ($>LC_{99}$) in
neonate larvae of *H. virescens*. Two of the compounds
30   studied were pyrethroids, allethrin (Type I pyrethroid)
or cypermethrin (Type II pyrethroid). In our study we
found a subadditive response when low rates of allethrin
or cypermethrin were combined with wild-type AcNPV.

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TABLE 4

Compound	n	μg Insecticide/Vial	% Mortality at 24 hours
Allethrin	121	0.05	14.0
Cypermethrin	110	0.008	11.8
DDT	80	0.02	18.8
Endosulfan	80	0.002	17.5
Methomyl	125	0.02	20.8
Profenofos	150	0.06	16.3

Lethal concentrations of insecticides (LC_{10}–LC_{20}) were chosen based on results from Campanhola & Plapp (1989).

Referring to Table 5 below, a combination of allethrin and wild-type AcNPV reduced the LT_{50} to 71.9 h (53.1 h, theoretical additive effect) which is a 17.9% reduction when compared to wild-type AcNPV alone. Likewise, cypermethrin in combination with wild-type AcNPV produced a LT_{50} of 65.8 h (52.1 h, theoretical) which represents a 24.2% reduction when compared to wild-type AcNPV. These results suggest that the pyrethroids and the wild-type AcNPV act independently of one another but result in a subadditive response since the combination is positive although it is significantly less than a theoretical interaction (additive effect).

The combination of the recombinant NPV, AcAaIT, with low rates of either pyrethroid produced a dose-response greater than an additive response. We observed that AcAaIT in combination with allethrin or cypermethrin resulted in 30.0 and 38.7% reductions in the LT_{50}s, respectively, when compared to AcAaIT alone. The LT_{50}s for the combination of AcAaIT and allethrin or cypermethrin were 40.2 h and 31.5 h. post infection, respectively (Table 5). Both of these LT_{50}s are lower than the theoretical (additive effect) LT_{50}s of 45.8 h and 47.2 h post infection, respectively. These data illustrate the synergistic effect.

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TABLE 5

Interactions of Recombinant or
Wild-Type AcNPV with Low Concentrations of Classical Insecticides

	Treatment[a]	Actual Kill Time (LT_{50}, h)	Theoretical Kill Time (h)[b]	% Reduction from WtAcNPV	% Reduction from RecNPV	Effect[c]
5	Wt AcNPV	70.2	---	21.8	---	---
	AcAaIT	89.8	---	---	---	---
	AcJHE.KK	76.2	---	15.0	---	---
	Allethrin	132	---	---	---	---
10	Allethrin + AcAaIT	40.2	45.8	55.2	30.0	Supplemental
	Allethrin + WtAcNPV	71.9	53.8	17.9	---	Subadditive
	Cypermethrin	124	---	---	---	---
15	Cypermethrin + AcAaIT	31.5	44.8	64.9	38.7	Supplemental
	Cypermethrin + AcJHE.KK	65.8	52.1	24.2	---	Subadditive
20	Cypermethrin + WtAcNPV	52.5	47.2	41.2	23.8	Subadditive
	DDT	96.7	---	---	---	---
	DDT + AcAaIT	41.2	40.8	54.1	29.0	Additive
	DDT + WtAcNPV	66.0	46.7	24.0	---	Subadditive
	Endosulfan	73.3	---	---	---	---
25	Endosulfan + AcAaIT	47.7	36.0	46.9	22.6	Subadditive
	Endosulfan + WtAcNPV	78.1	40.7	11.9	---	Antagonism
	Methomyl	358	---	---	---	---
30	Methomyl + AcAaIT	34.3	59.5	61.8	36.0	Supplemental
	Methomyl + WtAcNPV	50.3	71.9	39.5	---	Supplemental
35	Profenofos	401	---	---	---	---
	Profenofos + AcAaIT	53.2	59.9	40.8	17.0	Supplemental
	Profenofos + WtAcNPV	58.5	73.5	31.3	---	Supplemental

40   LT = lethal time; RecNPV = Recombinant NPV.

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^a Dose-response curves were developed for the classical insecticides using neonate larvae of *H. virescens*. Larvae infected with NPVs were exposed to 2000 polyhedrin inclusion bodies (PIBs; >LC_{99}) using a droplet feeding assay. Larvae were immediately transferred to vials treated with an approximate LC_{10}-LC_{20} (24 h) of the insecticide and scored every 8 to 12 h.

5   ^b Theoretical Kill Time = 1 ÷ [Reciprocal of LT_{50} for virus alone + Reciprocal of LT_{50} for insecticide alone].

^c Effect - Supplemental synergism is Actual Time > Theoretical Time; Additive synergism is Actual Time = Theoretical Time; Subadditive Synergism is Actual Time < Theoretical Time and Actual Time > Fastest Time Alone; Antagonism is Actual Time < Fastest Time Alone.

10   The foregoing examples illustrate certain embodiments of the present invention, and are not intended to limit the scope of the invention, which is defined in the appended claims.

30

It is Claimed:

1.   A method for controlling pests from the group insects, acarids, and nematodes comprising:
       treating said pests or their loci with a combination of recombinant insect virus and organic insecticide.

2.   The method as in claim 1 wherein the recombinant insect virus expresses a foreign protein or a functional derivative thereof in pest cells infected therewith.

3.   The method as in claim 2 wherein the expressed foreign protein or functional derivative is an insect toxin.

4.   The method as in claim 1 wherein the insect virus is a baculovirus.

5.   The method as in claim 4 wherein the baculovirus is a nuclear polyhedrosis virus.

6.   The method as in claim 5 wherein the baculovirus is from *Autographa californica*, *Anagrapha falcifera*, *Anticarsia gemmatalis*, *Buzura suppressuria*, *Cydia pomonella*, *Heliocoverpa zea*, *Heliothis arrigera*, *Mariestia brassicae*, *Plutella xylostella*, *Spodoptera exigua*, *Spodoptera littoralis*, or *Spodpotera litura*.

7.   The method as in claim 1 or 5 wherein the organic insecticide is a sodium channel agonist.

8.   The method as in claim 7 wherein the sodium channel agonist is a pyrethroid.

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9.   The method as in claim 1 or 5 wherein the organic insecticide is an acetylcholinesterase inhibitor.

10.   The method as in claim 9 wherein the acetylcholinesterase inhibitor is an organophosphate.

11.   The method as in claim 9 wherein the acetylcholinesterase inhibitor is a carbamate.

12.   The method as in claim 1 or 5 wherein the organic insecticide is a sodium channel blocking agent.

13.   The method as in claim 12 wherein the sodium channel blocking agent is pyrazoline.

14.   The method as in claim 1 or 5 wherein the organic insecticide is a nicotinic acetylcholine binding agent.

15.   The method as in claim 1 or 5 wherein the organic insecticide is a gabaergic binding agent.

16.   The method as in claim 1 or 5 wherein the organic insecticide is an octapamine agonist or antagonist.

17.   The method as in claim 1 or 5 wherein the organic insecticide is an oxphos uncoupler.

18.   The method as in claim 1 or 5 wherein the organic insecticide is an agent that binds to a cation channel.

19.   The method as in claim 18 wherein the organic insecticide is a peptide.

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20. A method of controlling insecticide
resistant pests comprising:

treating said pests or their loci with a
recombinant insect virus in conjunction with applying to
5   the loci thereof an amount of insecticide to which the
pest is resistant.

21. The method as in claim 20 wherein the
amount of insecticide is reduced with respect to a
determinable killing dose of insecticide when applied in
the absence of the virus.

22. The method as in claim 20 wherein the
insect virus is a baculovirus.

23. A method of treating insecticide
resistant pests comprising:

determining a first amount of insecticide to
which the pest is resistant, said first amount being
5   sufficient to kill at least about half of the insects;
and

administering a predetermined amount of said
insecticide to the loci of said pests in conjunction
with a recombinant baculovirus, the second amount being
10   lower than said first dose of the determining step.

24. The method as in claim 20 or 23 wherein
the insecticide is a pyrethroid.

25. The method as in claim 20, 23, or 24
wherein the baculovirus is a nuclear polyhedrosis virus.

26. The method as in claim 24 wherein the
nuclear polyhedrosis virus is *Autographa californica*.

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27.   The method as in claim 25 wherein the recombinant baculovirus expresses an insect-selective toxin.

28.   The method as in claim 26 wherein the toxin is from *Androctonus australis*.

29.   The method as in claim 27 wherein the pest is of the genera *Spodoptera*, *Trichoplusia*, or *Heliothis*.

30.   The method as in claim 27 wherein the pest is *Heliothis virescens* or *Heliocoverpa zea*.

31.   The method as in claim 29 wherein the insecticide is a pyrethroid.

AMENDED CLAIMS
[received by the International Bureau on 30 October 1995 (30.10.95);
original claims 1-3,17,20,21,23,25,27 amended;
new claims 32-35 added; remaining claims unchanged (5 pages)]

1.   A method for controlling pests from the group insects, acarids, and nematodes comprising:
treating said pests or their loci with a combination of recombinant insect virus expressing a foreign protein in cells of said pests infected therewith, and an organic insecticide, the combination effective to increase speed of pest kill.

2.   The method as in claim 1 wherein the foreign protein is an insect toxin or a functional derivative of the insect toxin that is active against said pests.

3.   The method as in claim 2 wherein the insect toxin is a scorpion toxin.

4.   The method as in claim 1 wherein the insect virus is a baculovirus.

5.   The method as in claim 4 wherein the baculovirus is a nuclear polyhedrosis virus.

6.   The method as in claim 5 wherein the baculovirus is from *Autographa californica*, *Anagrapha falcifera*, *Anticarsia gemmatalis*, *Buzura suppressuria*, *Cydia pomonella*, *Heliocoverpa zea*, *Heliothis arrigera*, *Mariestia brassicae*, *Plutella xylostella*, *Spodoptera exigua*, *Spodoptera littoralis*, or *Spodpotera litura*.

7.   The method as in claim 1 or 5 wherein the organic insecticide is a sodium channel agonist.

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8.   The method as in claim 7 wherein the sodium channel agonist is a pyrethroid.

9.   The method as in claim 1 or 5 wherein the organic insecticide is an acetylcholinesterase inhibitor.

10.   The method as in claim 9 wherein the acetylcholinesterase inhibitor is an organophosphate.

11.   The method as in claim 9 wherein the acetylcholinesterase inhibitor is a carbamate.

12.   The method as in claim 1 or 5 wherein the organic insecticide is a sodium channel blocking agent.

13.   The method as in claim 12 wherein the sodium channel blocking agent is pyrazoline.

14.   The method as in claim 1 or 5 wherein the organic insecticide is a nicotinic acetylcholine binding agent.

15.   The method as in claim 1 or 5 wherein the organic insecticide is a gabaergic binding agent.

16.   The method as in claim 1 or 5 wherein the organic insecticide is an octapamine agonist or antagonist.

17.   The method as in claim 1 or 5 wherein the organic insecticide is an oxidative phosphorylation uncoupler.

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18. The method as in claim 1 or 5 wherein the organic insecticide is an agent that binds to a cation channel.

19. The method as in claim 18 wherein the
5   organic insecticide is a peptide.

20. A method of controlling insecticide resistant pests comprising:
     treating said pests or their loci with a recombinant insect virus expressing a foreign protein in
10   cells of said pests infected therewith, in conjunction with applying to the loci thereof an amount of insecticide to which said pest is resistant, the amount of insecticide applied being not greater than the dose that would have been applied for nonresistant pests, the
15   recombinant insect virus and the insecticide effective together to increase speed of kill for said pest.

21. The method as in claim 20 wherein the speed of kill is faster for said pests than for the nonresistant pests.

20   22. The method as in claim 20 wherein the insect virus is a baculovirus.

23. A method of treating insecticide resistant pests comprising:
     determining a first amount of insecticide to
25   which the pest is resistant, said first amount being sufficient to kill at least about half of the insects; and
     administering a second amount of said insecticide to the loci of said pests in conjunction
30   with a recombinant baculovirus expressing a foreign protein in cells of said pests infected therewith, the

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second amount being lower than said first amount of the
determining step and the speed of kill being faster for
the insecticide and the recombinant baculovirus than for
the insecticide or the baculovirus alone.

5   24.   The method as in claim 20 or 23 wherein
the insecticide is a pyrethroid.

           25.   The method as in claim 23 wherein the
baculovirus is a nuclear polyhedrosis virus.

           26.   The method as in claim 24 wherein the
10   nuclear polyhedrosis virus is *Autographa californica*.

           27.   The method as in claim 25 wherein the
foreign protein is an insect-selective toxin.

           28.   The method as in claim 26 wherein the
toxin is from *Androctonus australis*.

15   29.   The method as in claim 27 wherein the
pest is of the genera *Spodoptera*, *Trichoplusia*, or
Heliothis.

           30.   The method as in claim 27 wherein the
pest is *Heliothis virescens* or *Heliocoverpa zea*.

20   31.   The method as in claim 29 wherein the
insecticide is a pyrethroid.

           32.   The method as in claim 1 or 5 wherein
speed of pest kill is faster than that set by a
theoretical combination kill time, C, wherein C is
25   defined by the relationship:
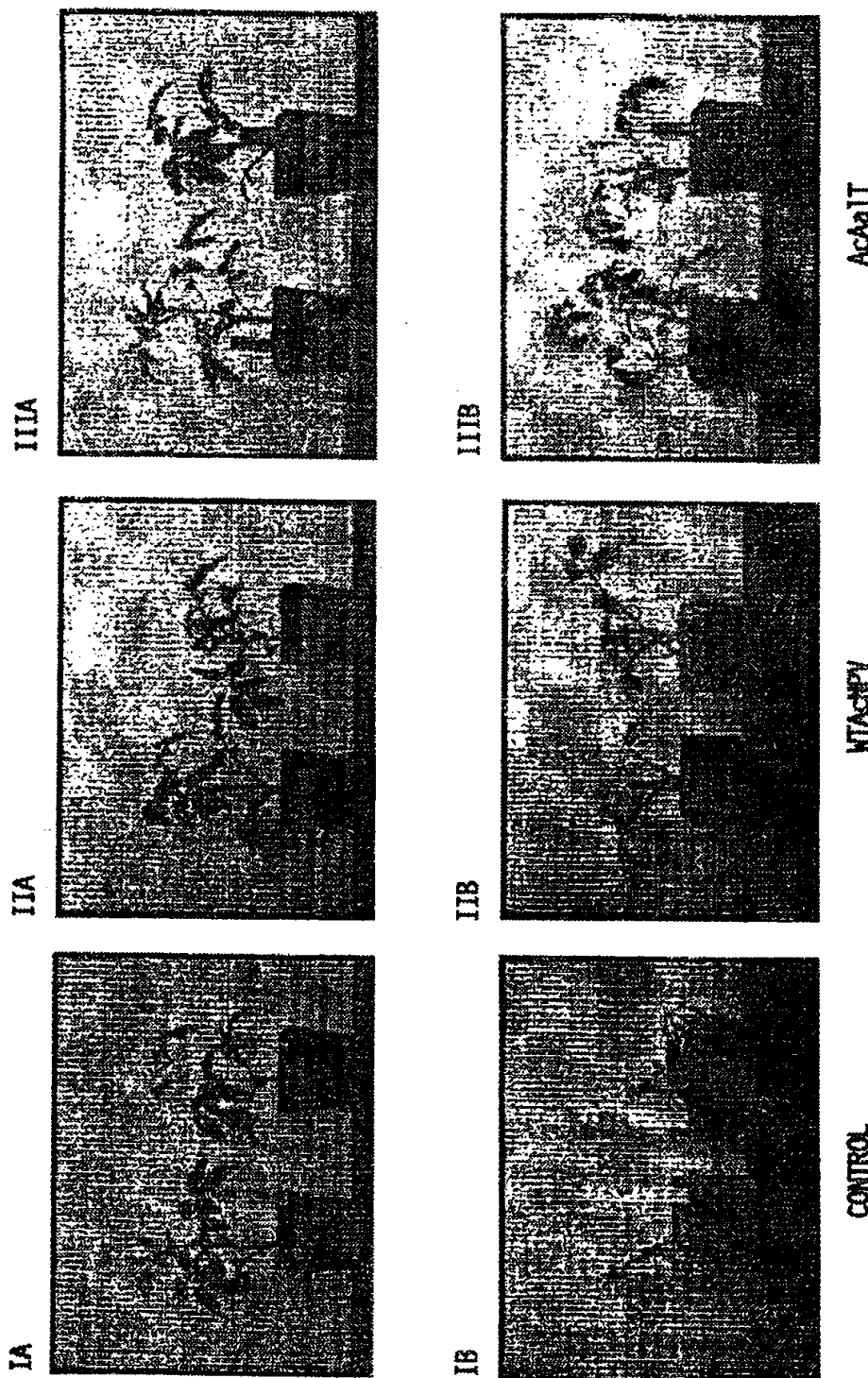
$$1/C = 1/A + 1/B$$

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wherein A is the individual kill time at a particular
concentration of recombinant insect virus and B is the
individual kill time at a particular concentration of
organic insecticide.

5   33. The method as in claim 5 wherein the
baculovirus is the nuclear polyhedrosis virus *Autographa
californica* expressing the toxin from *Androctonus
australis* and the insecticide is a pyrethroid or an
acethycholinesterase inhibitor.

10   34. The method as in claim 20 wherein the
insect   virus   is   the   nuclear   polyhedrosis   virus
Autographa californica expressing a toxin from
Androctonus australis and the insecticide is a
pyrethroid.

15   35. The method as in claim 23 wherein the
baculovirus is the nuclear polyhedrosis virus *Autographa
californica* expressing a toxin from *Androctonus
australis* and the insecticide is a pyrethroid.

AMENDED SHEET (ARTICLE 19)

FIG. 1

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A01N 63/00; 27/00; C12N 7/01

US CL :424/93.2, 93.6, 199.1, 204.1, 405; 435/235.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/93.2, 93.6, 199.1, 204.1, 405
435/172.3, 235.1, 240.2, 320.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 4,668,511 (ASPIROT *ET AL.*) issued 26 May 1987, see entire document.	1-31
Y	US, A, 5,238,724 (BJOSTAD *ET AL.*) issued 24 August 1993, see entire document.	1-31
Y	US, A, 5,162,308 (BROWN *ET AL.*) issued 10 November 1992, see entire document.	1-31
Y	US, A, 4,888,340 (NEH *ET AL.*) issued 19 December 1989, see entire document.	1-31
Y	BIO/TECHNOLOGY, Volume 9, issued September 1991, McCutchen et al., "Development Of A Recombinant Baculovirus Expressing An Insect-Selective Neurotoxin: Potential For Pest Control", pages 848-852, see entire document.	1-31

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance		
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
03 JULY 1995	28 AUG 1995

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INTERNATIONAL SEARCH REPORT

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Science, Volume 261, issued 30 July 1993, Moffat, "New Chemicals Seek To Outwit Insect Pests", pages 550-551, see entire document.	1-31

B. FIELDS SEARCHED-

Electronic data bases consulted (Name of data base and where practicable terms used):

Automated Patent System - USPAT and JPOABS
DIALOG - BIOSIS
Search Terms: insecticide, pesticide, baculovirus, toxin, pyrethrin, carbamate, organophosporus, pyrazoline, imidacloprid, formamidine, Autographica,